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December 23, 2013

To Whom It May Concern:

On behalf of Dr. Tracy Romano, please find reporting documents for Office of Naval Research award N00014-11-1-0437, as required for grant closeout. Please contact me with any questions.

Sincerely,

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The objectives of this effort are: 1) To monitor the neuroimmunoendocrinological responses of three resident aquarium belugas before and after the introduction and throughout the adaptation process of seven new belugas to their habitat and to measure the neuroimmunoendocrinological responses of 5 belugas to transport and 2) To monitor the neuroimmunoendocrinological responses (via blood, saliva, blow and feces) of 2-3 aquarium resident whales before and after the occurrence of a known stressor (i.e. an out of water examination).

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Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health

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LONG-TERM GOALS

The overall top level goal of this effort is to investigate the physiological i.e. neuroimmunoendocrinological responses of beluga whales to "stressors". "Stressor events" will allow for a better understanding and characterization of the relationships among hormones (e.g. cortisol, corticosterone, adrenocorticotropin hormone, aldosterone, catecholamines) in different matrices (blood, saliva, blow, feces) in conjunction with immune function. In addition, "stressor events" will enable us to define and compare the quantitative and temporal relationships of hormones across different matrices.

OBJECTIVES

The objectives of this effort are: 1) To monitor the neuroimmunoendocrinological responses of three resident aquarium belugas before and after the introduction and throughout the adaptation process of seven new belugas to their habitat and to measure the neuroimmunoendocrinological responses of 5 belugas to transport and 2) To monitor the neuroimmunoendocrinological responses (via blood, saliva, blow and feces) of 2-3 aquarium resident whales before and after the occurrence of a known stressor (i.e. an out of water examination (OWE)).

APPROACH

Seven belugas, *Delphinapterus leucas* (one male 22 years of age, four females 9-26 years of age, two calves < 2yrs, 1 male and 1 female) were transported from Shedd Aquarium, Chicago, IL to Mystic Aquarium, Mystic, CT in the fall of 2008 and remained at Mystic Aquarium until the spring of 2009 while exhibit modifications occurred at the Shedd Aquarium. The transported belugas were initially placed into a holding pool physically separated from the three resident belugas (one male and two

females approximately 27 yrs) at Mystic Aquarium. However, all belugas could establish visual and auditory contact with each other. Blood samples had been collected and archived for a subset of the transported belugas at time points before, during and after the transport and introduction. Catecholamines will be measured in blood via High Performance Liquid Chromatography; adrenocorticocotropin hormone (ACTH), cortisol and aldosterone will be measured via established chemiluminescent and radioimmunoassays at the Animal Health Diagnostic Center, Endocrinology, College of Veterinary Medicine or via enzyme immunoassay (EIA) at the Mystic Aquarium; immune function including the ability of lymphocytes to proliferate, quantification of lymphocyte subsets and phagocytosis and respiratory burst of neutrophils and monocytes (via flow cytometry) will be assessed. The relationships among hormones before after the stressors will be evaluated as well as the relationships between the hormones and immune function.

To fulfill objective 2, samples of blood, saliva, blow and feces will be collected at time points before, during and after out of water examinations (OWEs) for 2-3aquarium resident whales. Catecholamines, ACTH, cortisol, aldosterone and immune function will be measured as above. Methodology for quantification of cortisol or corticosterone in beluga blow, saliva, and feces will be developed. The relationships among hormones before and after the stressors will be evaluated as well as the relationships between the hormones and immune function. The quantitative and temporal relationships of corticosteroid hormones across the different matrices will be evaluated.

Tracy Romano (P.I.) is primarily responsible for overseeing all sample collection and analyses, the development and transition of hormonal assays, and data integration and analyses. She will coordinate the project with the Co-Investigators both (on-site and off-site), she will write the results of the research in manuscript format for publication and present the research at scientific meetings and public forums.

Tracey Spoon (Co-PI) passed away in May 2012. Mandy Keogh assumed her role as Co-P.I. on the project in September, 2012.

Mandy Keogh (Co-P.I.) will play an activate role in sample collection, assay development and coordination of sample analyses in the laboratory, and work closely with the technician and graduate student on the analyses in the laboratory. She will work with the P.I. on data integration and analysis as well as publication.

Steve Lamb (Co-P.I.) will be responsible for the ACTH, aldosterone and cortisol assays that will be conducted at the AHDC at Cornell and advise and assist the P.I. and Co-P.I. with hormonal assay development and validation and transition of technology to Mystic Aquarium.

Laura Thompson, PhD student at the University of Connecticut (UCONN), Department of Marine Biosciences will contribute to development of an assay for measuring stress hormones in cetacean blow as well as play a role in carrying out the immune function assays in the laboratory and assist with sample collection and data analyses.

WORK COMPLETED

Objective 1: Complete blood cell counts including total white blood cell (WBC) and differential couts along with phagocytosis and respiratory burst activity of granulocytes and monocytes were assayed immediately following blood collection. Hormone (cortisol, ACTH, and aldosterone) and

catecholamine analysis (epinephrine, norepinephrine and dopamine) have been completed. Results on the influence of translocation and introduction to a novel environment on the innate immune system, as measured by phagocytic and respiratory burst activity has been published (Spoon and Romano, 2012). Assays for immunophenotyping of lymphocyte subpopulations have been completed. Further, experiements have been completed to determine the optimal parameters for the lymphocyte proliferation assay (LPA) in belugas. LPAs have been carried out on many of the archived samples; however some analyses are pending. It is expected that these results with the catecholamine and hormone data will be published in a similar manner as the previous manuscript focused on the innate response.

Objective 2: Behaviors for blood sampling, blow, saliva and feces collection are under continuous reinforcement on Aquarium belugas. Monthly sampling and archiving have occurred for these tissue matrices. To date, a total of six OWEs have been conducted on three whales (2 female, 1 male). One female beluga completed one OWE and subsequent OWEs were canceled due to phlebitis of the fluke vessels, adverse weather conditions, as well as an illness unrelated to the current ONR project. The male beluga has completed two OWEs and the remaining female whale participated in three OWEs; however, time points for blood sampling were fewer due to prior incidence of phlebitis and pre and post samples were limited due to a breakdown in behaviors. While blood samples were not collected under behavioral control, the other biological samples (blow, saliva, feces) were attempted before and after the OWEs.

Complete blood cell counts along with phagocytosis and respiratory burst activity of granulocytes and monocytes were assayed immediately following blood collection. All archived samples have been analyzed for catecholamines and all samples submitted to Cornell for analysis of cortisol, ACTH, and aldosterone with results from remaining tests expected within the next two weeks. LPAs have been completed for all samples from the six OWEs. Immunophenotyping of the lymphocyte subpopulations associated with all but the 2013 OWEs have been completed. Those for 2013 are currently underway.

Protocols for optimal collection and processing of blow, saliva and blood have been developed. Five commercially available cortisol immunoassay kits were tested for use in quantifying cortisol in beluga blow and saliva samples (Salimetrics, MP Biomedical, Enzo Life Sciences, Caymen and Arbor Assays). A pool of blow, saliva and plasma samples from 4 beluga whales were used to test these specific matrices with the cortisol EIA kits. The final decision on which kit to use was based on the best results for all matrices. Additional tests were carried out on the chosen kit for intra- and inter-assay variability as well as interferring substances utilzing a charcoal stripping technique.

Fecal samples have been continually collected in association with monthly blood draws and OWEs. To date fecal samples have been dried and sifted through a mesh, stirred and stored at -80°C until validation of a commercially available kit for measuring corticoserone and/or cortisol in beluga feces.

There were some major challenges throughout the project. The Co-PI on the project, Dr. Tracey Spoon passed away unexpectedly in early May, 2012. There was a lag time before this position was replaced and the new CoPI up and running on the project. Also, there were issues with completion of the full number of OWEs. Phlebitis occurred in two of the three whales and took a long time to resolve. In addition there was a breakdown of behaviors during breeding season which were not fully regained, as well as other health issues. Moreover, there were only short time periods in which the OWEs could be conducted due to temperature and weather.

RESULTS

Objective 1: Results on the influence of translocation and introduction to a novel environment on the innate immune system, as measured by phagocytic and respiratory burst activity has been published (Spoon and Romano, 2012). Briefly, transported belugas exhibited increases in catecholamines and cortisol and an increase in phagocytosis and respiratory burst activity while resident whales showed an increase in catecholamines but not cortisol after the introduction of the transported whales and a decrease in neutrophil and monocyte function. Resident belugas showed a decrease in circulating lymphocytes upon introduction of the translocated whales as did the translocated whales. Experiments have been completed to determine the optimal parameters for the LPA in belugas. The optimal parameters for carrying out the lymphocyte proliferation assay included the following: 1×10^5 cells/well, a total incubation period of 96 hr, and optimal and suboptimal mitogen concentrations at 2.5 and 1.25 µg/ml for ConA and 25 and 2.5 µg/ml for LPS. The analyses on the immunophenotyping and the remaining assays and analyses for the LPA are currently being completed.

Objective 2: The following results were obtained for optimal collection, processing and analysis for cortisol quantification in blow, saliva and feces:

Blow Preliminary experiments investigated different collection devices (50 ml conical tubes, 250 ml Nalgene™ bottles, Petri dishes), different collection materials (cotton gauze, nylon stocking, tulle, Nitex membrane), and number of exhales needed from whales to give an appropriate volume for assay. The Petri dish covered with Nitex membrane provided the best results as did 4-8 repeated exhales. Five commercially available cortisol EIA kits were tested. The assay from Cayman Chemical was chosen for further validation for use with blow samples based on preliminary results. Kit validation included parallelism and linearity for separate pools of male and female blow (Figure 1). Serially diluting pooled samples 1:2, 1:4, 1:8, and 1:16, resulted in curves with good linearity, slopes approximating 1, and parallelism with the standard curve.

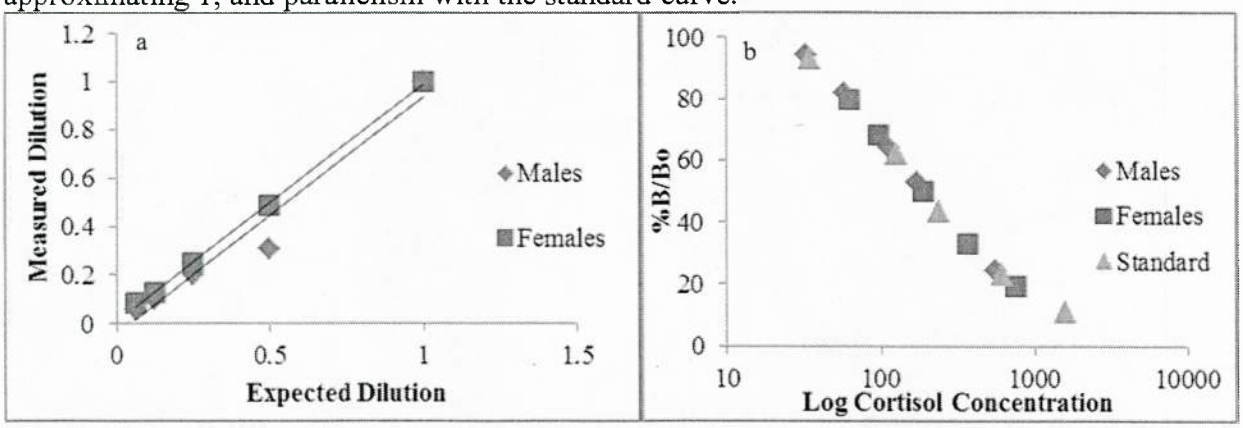

Figure 1 a) Linearity testing for blow samples pooled between males (y=0.9857x-0.0469; r2=0.9573) and females(y=0.9843x+0.0077; r2=0.9991). b) Log cortisol concentration plotted against the % B/Bo for the kit standards and for blow pooled between males and females.

In order to test accuracy and recovery, both pooled blow samples as well as the Nitex collection material were spiked with kit provided standards and tested for recovery. Four standards (41, 102.4, 256, and 640 pg ml^{-1}) were spiked into pooled blow samples. Cortisol measured in unspiked blow was subtracted from the cortisol measured in spiked blow and the resulting concentration of cortisol was

compared with an expected value (Figure 2). Slopes of these lines showed an approximation to 1. Additionally, three standards (102.4, 256, and 640 pg ml^{-1}) were used to spike collection material, and showed 80-110% recovery.

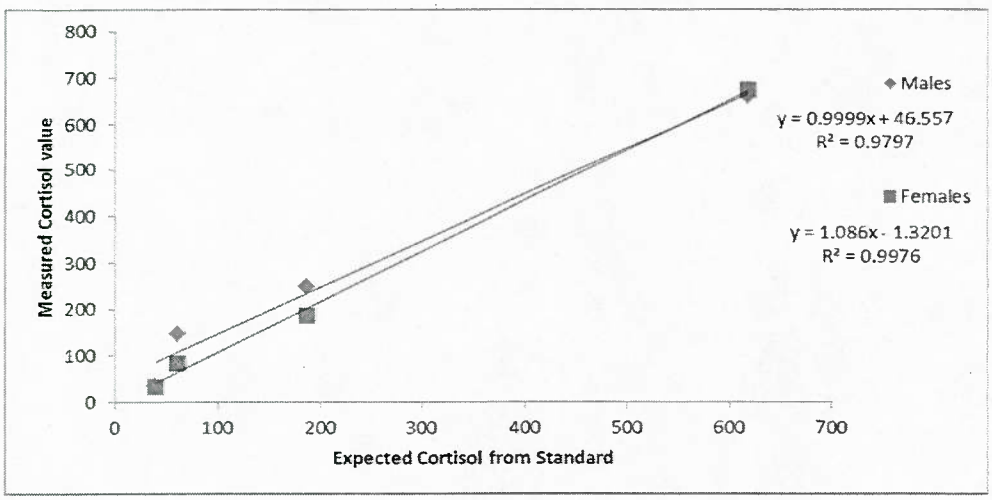

Figure 2. Expected and measured cortisol values from pooled blow samples spiked with kit provided cortisol standards. Slopes approximate 1 for both males (y=0.9999x + 46.557; r2=0.9797) and females (y=1.086x – 1.3201; r2=0.9976).

Based on the above results archived blow samples were measured with the above assay and plasma samples were submitted to Cornell. Blow samples were obtained in conjunction with blood prior to, during and following OWEs. Cortisol values in blow and blood from samples taken at baseline, 30 minutes on the beach and 24 hours post release showed an increase during the OWE with values returning to near baseline 24 hours later. Although cortisol in blow followed the same trends as cortisol in blood, only plasma samples were statistically significant (Table 1).

Table 1: Average cortisol values for three belugas at baseline, 30 minutes out of the water and 24 hours post release for the OWE (α =0.05).

		Baseline	30minOWE	24hrsPost	Repeated Measures ANOVA
Cortisol $\mu g\ dl^{-1}$	Blow	0.13 ± 0.09	0.38 ± 0.41	0.07 ± 0.02	F=1.177 p=0.396
	Plasma	1.62 ± 0.78	7.97 ± 2.08	2.10 ± 1.40	F=19.953 p= 0.032

A manuscript has been written and will be submitted to General and Comparative Endocrinology describing the methodology for collection, processing and measurement of cortisol in beluga blow within the next week.

Saliva

As with blow, preliminary experiments were conducted on saliva collection, materials and processing. Initial linearity and parallelism testing for saliva suggested that the Cayman Chemical kit was also a good fit for measuring cortisol in this sample matrix. However, recovery and accuracy testing

suggested that there was gross overestimation of cortisol in samples (up to 200% recovery). Further testing suggested that this issue stemmed from incompatibility between the collection material and the kit. An alternative cortisol kit was then chosen for testing. Recovery testing revealed excellent agreement between measured and expected cortisol values. However linearity testing revealed very low cortisol content of pooled samples (< 0.01 $\mu g\ dl^{-1}$) with B/Bo values > 80% with the majority being 90% and greater. In an attempt to concentrate samples, SiliaPrep C18, 50 mg/1 ml SPE cartridges were purchased from Silicycle (Quebec City, Quebec, Canada). Linearity testing was rerun, however the results were similar with high B/Bo values suggesting very low cortisol content. Further tests are needed to determine if EIA is suitable for measuring cortisol in saliva or if radioimmunoassay is needed.

Out of Water Events

A total of six OWEs have been conducted on three whales (2 female, 1 male). Catecholamine and hormone concentrations in blood from each time point have been quantified. Results from the last 2 have been submitted for analysis but are currently pending. During the first OWE, only cortisol and ACTH were significantly elevated while the animals were beached out of water (Table 2).

Table 2. Mean, standard deviation and statistics for selected hormones and catecholamines collected from 3 belugas during an OWE.

	Prior	Beach	Post	Repeated Measure ANOVA
Cortisol	2.4 ± 1.3	7.4 ± 1.9	2.0 ± 0.6	F=24.96, p=0.006
ACTH	11.1 ± 5.1	23.7 ± 5.3	10.2 ± 7.2	F=74.941, p=0.001
Norepinephrine	685.1 ± 139.3	628.7 ± 229.6	487.5 ± 139.6	F=0.902, p=0.475

Complete blood cell counts along with phagocytosis and respiratory burst of granulocytes and monocytes were assayed immediately following blood collection for each OWE and statistical analysis of the results are currently underway. LPA and immunophenotyping of lymphocyte subsets have also been carried out on all samples with data analysis on the more recent OWEs pending. Figure 3 shows the results for LPA for a beluga during an OWE, with a decrease in response 48 hours post OWE and results for lymphocyte subsets are shown in Figure 4 with a similar trend for most subsets.

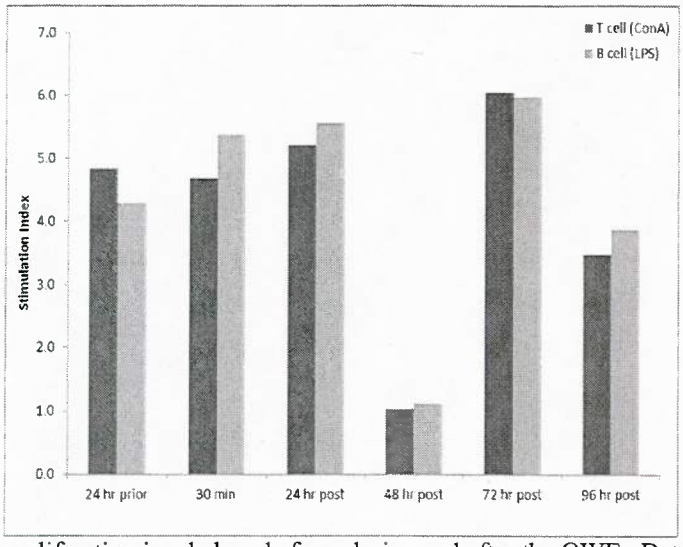

Figure 3. Lymphocyte proliferation in a beluga before, during and after the OWE. Data are presented as a stimulation index (SI; mean OD of cells exposed to mitogen/mean OD of cells in media only).

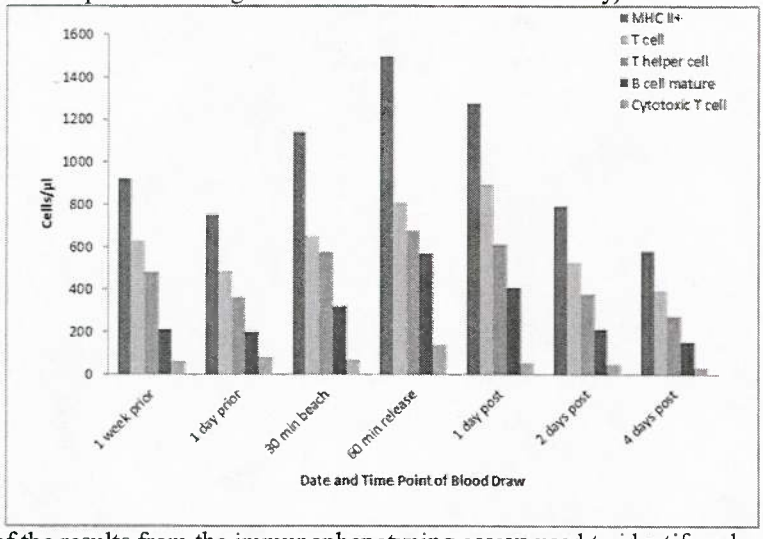

Figure 4. An example of the results from the immunophenotyping assays used to identify subpopulations of lymphocytes for one resident beluga prior to, during and following the first out of water experience.

In summary the results from these studies show that transport, introduction to novel social groups, and OWEs brought about changes in the neuroimmunoendocrinological responses of belugas that are measurable and quantifiable. Moreover, techniques have been developed and validated for collection, processing and measurement of cortisol in blow and to some extent in saliva, two alternative non-invasive sampling matrices. The magnitude of the responses are different depending on the stressors as well as for the individuals themselves. Pending analyses of results will be integrated with current analyses for a complete analysis of stress hormones and immune response in blood and other tissue matrices following specific stressors in belugas.

IMPACT/APPLICATIONS

There is increasing concern regarding the potential effects of anthropogenic sound on marine mammals. The U.S. Navy is under continuous scrutiny with regards to sonar exercises and impacts on marine mammals. While studies have been conducted on behavioral and auditory responses in marine mammals with respect to anthropogenic sound there is a lack of scientific data and knowledge of the

physiological impacts of loud sound exposure on marine mammals. There are many limitations and constraints in investigating the effects of anthropogenic sound as a "stressor" and impacts on the physiology of marine mammals. Despite these limitations there is a recognized need for such studies.

Investigation of the physiological response to stressors is very difficult in marine mammals given the difficulty in imposing stressors on marine mammals that will elicit a response, the lack of validated assays for measuring stress hormones, the difficulty in obtaining samples without causing stress, and obtaining a large enough sample size to draw significant conclusions. We are uniquely positioned at Mystic Aquarium, a division of Sea Research Foundation, Inc. to overcome some of the above obstacles and can provide a better understanding of the relationships among hormones in different matrices and in relation to immune function after stressor events. We can also define and compare the quantitative and temporal relationships of hormones across different matrices. This basic information is needed to lay the groundwork for understanding the impact of anthropogenic sound on marine mammals individually and at the population level.

RELATED PROJECTS

Title: Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population
PI: Dorian Houser, PhD National Marine Mammal Foundation
Longitudinal study of a large dolphin population to characterize stress markers in different matrices. Proposed effort also includes investigating the responsiveness of the thyroid and corticosteroid hormone production pathways.

Title: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (*Tursiops truncatus*)
PI: Pat Fair, PhD National Ocean Service
Study investigating hormones and immune function in wild dolphins vs. two different populations of managed-care dolphins (Aquarium setting vs. San Diego Bay).

Title: Baseline Health Measurements in Wild Belugas
PI: Tracy Romano, PhD Mystic Aquarium, a division of Sea Research Foundation
Study investigating hormones and immune function in wild beluga whales.

PUBLICATIONS

Spoon, T.R. and T.A. Romano. 2012. Neuroimmunological Response of Beluga Whales (*Delphinapterus leucas*) to Translocation and a Novel Social Environment. Brain, Behavior and Immunity 26:121-131.

Thompson, L.A., Spoon, T.R., Goertz, C.E., Hobbs, R.C., Romano, T.A. Blow Collection as a Non-Invasive Method for Measuring Cortisol in the Beluga (*Delphinapterus leucas*). Gen. Comp. Endocrinol. *Submitted.*